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**Characterization of a Novel Long Non-coding RNA Involved in  
Thyroid Differentiation**

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## Abstract

Thyroid is the endocrine gland that most frequently undergoes to congenital disorders or neoplastic transformation and despite its organogenesis is well characterized, molecular bases of early thyroid differentiation are still obscure. During last years, long non-coding RNAs (lncRNA) have acquired increasing relevance in many biological processes, such as differentiation and cancer. In E10.5 mouse thyroid bud the most enriched transcript resulted to be a poorly characterized lncRNA, that we named Thybe1 (thyroid bud enriched 1). Thybe1 is an antisense transcript of the protein-coding gene *klhl14*, also enriched in thyroid bud, to which it partially overlaps in a head-to-head arrangement. To shed light on its role, in this work we characterize such novel lncRNA, investigating its role in thyroid differentiation and its possible mechanism of action. Interestingly, we observed that Thybe1 is dramatically repressed during thyroid carcinogenesis, being inversely correlated with miR182a-5p expression, both *in vitro* and *in vivo*. Furthermore, we noted that Thybe1 expression positively correlates with that of *Klhl14* in several cell types, suggesting that this lncRNA could also be able to act *in cis* on this target. In conclusion we describe for the first time a lncRNA involved in thyroid differentiation and carcinogenesis, and start to highlight its ability to act as a competing endogenous RNA for key developmental genes, thus identifying a novel candidate gene playing a role in thyroid development defects and cancer.

# 1 Background

## 1.2 *Long non-coding RNAs play relevant roles in many biological processes*

### 1.1.1 *Long non-coding RNAs*

In recent years, it has been becoming clear that transcribed but untranslated portion of eukaryotes genomes exceeds the protein-coding one (Maeda et al., 2006; Katayama et al., 2005, Diez-Roux et al., 2011; Djebali et al., 2012; Costa 2010), hinting organism complexity could mainly depend on a RNA-mediated regulatory mechanism (Fatica and Bozzoni, 2014; Morlando et al., 2014). Non-coding RNA is a high wide class of molecules that are mainly classified according to their length in small and long non-coding RNAs (lncRNA).

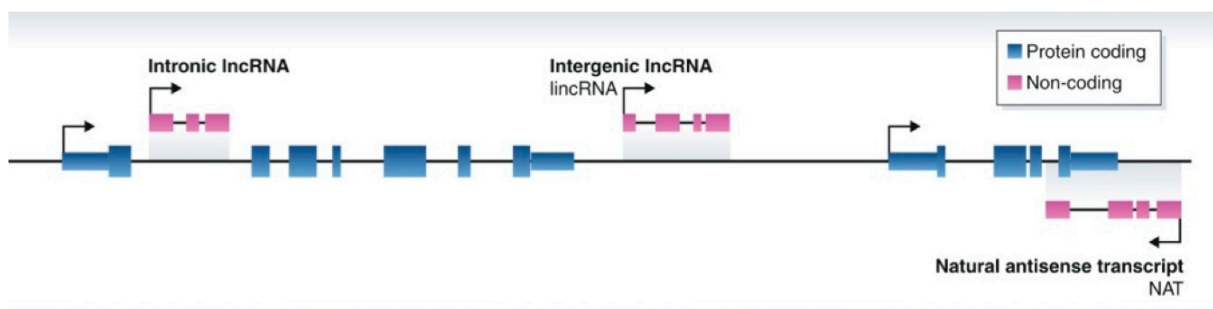
LncRNAs are RNA molecules longer than 200 nucleotides and without an evident open reading frame. They often are 5' capped, polyadenylated and alternatively spliced (Guttman et al., 2009; Derrien et al., 2012). The number and the relevance of this kind of transcripts have been increasing, in last years, revealing their manifold functions. Despite some common features, lncRNAs are a very heterogeneous group of transcripts that could be classified according to their genome location or their functions.

LncRNAs could be divided in:

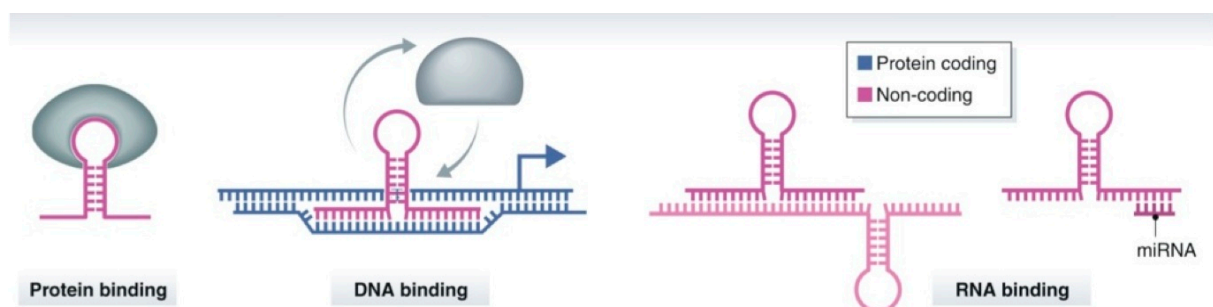
- lincRNAs: long intergenic non-coding RNAs are located between two protein-coding or non-coding genes, to which it does not overlaps.
- Intronic lncRNAs: These RNAs are transcribed from genes situated within the introns of protein-coding genes
- NATs: Natural Antisense Transcripts are transcribed in the opposite sense of a coding gene located on the other strand. The two genes could partially overlap (Scheuermann and Boyer, 2013) (Figure 1).

LncRNAs can play different roles because they can act in different ways. Being RNA molecules, lncRNAs are able to bind both DNA, RNA and proteins. Moreover, thanks to their length, these molecules can fold into secondary structures that allow them to improve the interaction with protein complexes and/or DNA and others RNAs (Figure 2)(Wilsuz et al., 2009, Guttman et al., 2012; Scheuermann and Boyer, 2013; Marchese and Huarte, 2014;

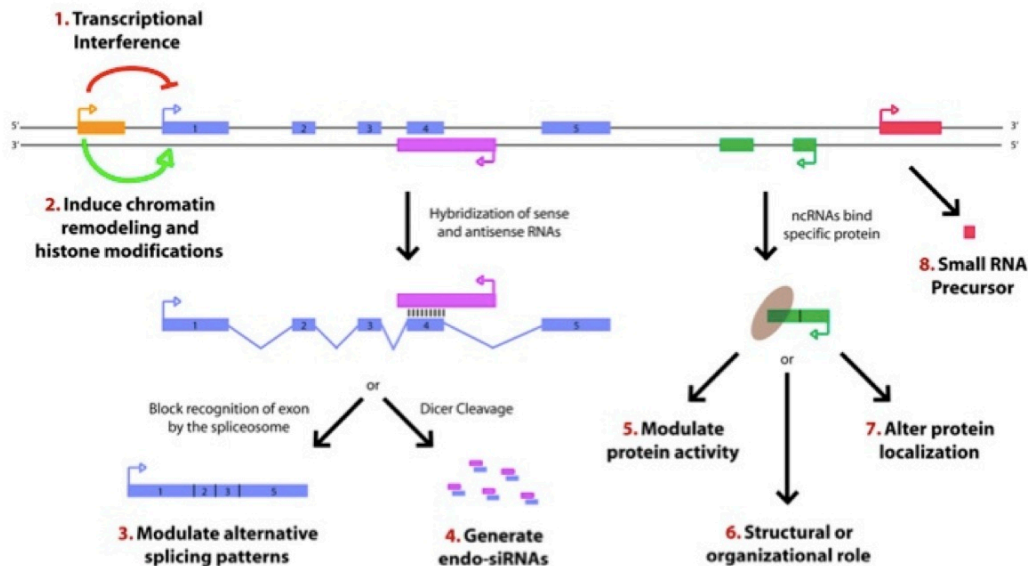
Fatica and Bozzoni, 2014). LncRNAs can act as signals molecules, decoy, guide or scaffold for transcription factors or chromatin-modifying complexes, modulator of protein activity or splicing process, precursors of small RNAs and sponge for microRNAs (Figure 3). (Wilsuz et al. 2009; Wang and Chang, 2011, Marchese and Huarte, 2014; Salmena et al., 2011) Hence, they are implicated in transcriptional and epigenetic regulation of their targets (Costa, 2010), but they are also able to modulate their target levels in a post-transcriptional manner. Indeed, acting as a competing RNA for miRNA binding, lncRNAs can regulate the expression of mRNAs that are targets of the same miRNA. (Salmena et al., 2011) It was also demonstrated they can act in cis, to regulate neighbour genes, or in trans, on their distant targets (Zucchelli et al., 2015; Scheuermann and Boyer 2013; Vance et al., 2014; Boque-Sastre et al., 2015; Morlando et al., 2014). All these functions, from transcriptional to epigenetic control, allow lncRNAs to modulate their targets expression (Morris, 2009; Ørom et al., 2010), being involved in many important biological event, such as differentiation, development and so, several different diseases and cancer.



**Figure 1. Representative class of long non-coding RNAs based on genomic location.** LncRNAs can be located within introns of protein-coding genes (intronic lncRNAs, on the left) or between two coding genes and so called intergenic lncRNAs (lincRNAs, in the middle) without overlapping with their exons. Then they can be located on the opposite strand of a coding or non-coding gene and transcribed in the antisense direction with a partially overlapping with its exons. These RNAs are called Natural Antisense Transcripts (NATs, on the right) (Scheruermann and Boyer, 2013)



**Figure 2. Global mechanisms of lncRNAs.** Thanks to their structure lncRNAs are able to interact with proteins (left), bind DNA (middle) and RNA molecules such as mRNAs or microRNAs (right). (Scheruermann and Boyer, 2013)



**Figure 3. Representative scheme of lncRNAs functions.** Transcription from an upstream non-coding promoter (orange) can negatively (1) or positively (2) affect the expression of downstream gene (blue) by inhibiting Pol II recruitment or inducing chromatin remodelling respectively. (3) An antisense transcript (purple) is able to hybridize to the overlapping sense transcript (blue) and block recognition of the splice sites by the spliceosome, thus resulting in an alternatively spliced transcript. (4) Alternatively, hybridization of the sense and antisense transcripts can allow Dicer to generate endogenous siRNAs. By binding to specific protein partners, a noncoding transcript (green) can modulate the activity of the protein (5), serve as a structural component that allows a larger RNA–protein complex to form (6), or alter where the protein localizes in the cell (7). (8) Long ncRNAs (pink) can be processed to yield small RNAs, such as miRNAs (Wilsuz et al., 2009)

### 1.2.1 Long non-coding RNAs and organ differentiation

Long non coding RNAs have been described to be expressed at lower levels than protein-coding genes and in a more tissue and cellular specific manner (Cabili et al., 2011; Morlando et al., 2014; Marques and Ponting, 2009; Boque-Sastre et al., 2015; Vučićević D, 20015). This specificity suggests their involvement in determining cell fate, acting as regulators of many differentiation processes (Flynn and Chang, 2014). Some lncRNAs operate modulating their targets transcription. For example, the lncRNAs Braveheart and Fendrr were described to be specifically required for cardiomyocytes development, acting as a decoy for the

Polycomb repressive complex PCR2 (Scheuermann and Boyer, 2013). PCR2 decoy is exploited also by the lncRNA-N1 and lncRNA-N3 in driving neurogenesis. Neurogenesis is the differentiation process that implicates the major number of lncRNA, probably for the high number of different cell type it has to generate (Fatica and Bozzoni, 2014). One of the central lncRNA involved in this process is Evf2 (also known as Dlx6os1). It negatively regulates, in cis, Dlx6 expression whereas recruits, in trans, the transcription activator factor DLX2 and the repressor MECP2 to regulate the expression of Dlx5 and Gad1. Evf2 mutants show defects in synaptic inhibition, hinting the important role this lncRNA play in neuronal activity (Feng et al., 2006)

However, despite transcriptional regulation is a well described and very common mechanism of action for lncRNAs, these molecules work in many other manners. The lncRNA TINCR, for example, promotes keratinocytes differentiation stabilizing the targeted mRNAs, such as keratin 80 (Fatica and Bozzoni, 2014)

During myogenesis, instead, linc-MD1 competes for mir133b and mir135 binding with the key myoblast differentiation factors MALM1 and MEF2C. Through this miRNAs sponge activity, linc-MD1 presence increases MALM1 and MEF2C expression, improving myogenesis (Cesana et al., 2011; Twayana et al., 2013).

This evidence highlights the heterogeneous range of roles lncRNAs are able to play and their relevance in specific tissue differentiation programs.

### **1.2.2 Long non-coding RNAs in cancer.**

Considering their involvement in differentiation processes, it was rational to conjecture that lncRNAs were also implicated in the loss of differentiation and then in carcinogenesis. Indeed, several lncRNAs have been described as involved cancer onset and/or progression. In AML cells, for example, many lncRNAs resulted involved in granulocytic differentiation (Hughes et al., 2014). Among these, UCA1 was classified as oncogenic because it supports the proliferation of AML C/EBP $\alpha$  mutated cells by repressing the cell cycle regulator p27kip1 (Hughes et al., 2015). Many lncRNAs are described as up-regulated in different type of cancer, causing the repression of their targets interacting with the Polycomb repressive complex PRC1/2. Among these, TUG1, for example, is up-regulated in lung cancer; MALAT1 is associated with malignancy of liver, breast and colon cancer (Huarte, 2015; Wang et al., 2017) and HOTAIR, is known to promote metastasis in liver, breast and

lung cancers, being implicated in epithelial-to mesenchymal transition (EMT)(Wang et al., 2017, Huarte, 2015; Gibb et al., 2011).

However, interacting with protein complexes is not the only mechanism through which lncRNAs modulates cancer aggressiveness. Indeed, the lncRNA ZFAS1 competes for the miR-150-5p binding to regulate Sp1 expression in ovarian cancer. ZFAS1 dysregulation has been observed in breast, colorectal and gastric cancer and hepatocellular carcinoma and it is associated with a poor prognosis of ovarian cancer (Xia et al., 2017). The miRNA-mediated mode of action was also exploited by lncRNA HULC that is high expressed in primary liver tumors and is considered a biomarker of liver cancer (Gibb et al., 2011). As well as ZFAS1, HULC, MALAT1 and the antisense transcript TRPM2, together with PCA3, are currently used as prognostic markers of liver, lung and prostate cancer respectively (Huarte 2015; Gibb et al., 2011; Xia et al., 2017; Orfanelli et al., 2015). Moreover, the NAT of Zeb2 gene has been described to be associated with cancer with low levels of E-cadherin. This natural antisense transcript positively regulates the transcriptional repressor Zeb2, leading to E-cadherin reduction and so promoting EMT (Sanchez and Huarte, 2013).

Given their overexpression and functions observed in cancer, these lncRNAs are currently considered as oncogenic RNAs, whereas lncRNAs as GAS5 and XIST are classified as tumor suppressors. Indeed, XIST expression is considerably reduced in breast cancer comparing with normal tissue and GAS5 is down-regulated in multiple cancers, such as breast, colorectal, lung, prostate and ovarian cancers. It is involved in cell cycle regulation, as described in neuroblastoma and acts as sponge for miR-21 in breast cancer (Wang et al., 2017; Li et al., 2016; Mazar et al., 2016).

Because of their regulatory functions, lncRNAs often mark the early phases of carcinogenesis, so they could be very useful in the prognosis of cancer progression but also in developing new therapeutic approaches. Indeed, given the relevance these molecules have been acquiring in cancer biology in last years, the interest in exploiting lncRNAs as new therapeutic targets is also increasing. Several strategies have been tested to target the RNA of interest, such as nanoparticles, peptides-mediated delivery and CRISP/Cas9 genome editing. However, handling RNAs in vivo presents many hurdles that it is need to overcome, such as the innate immunity, the target selectivity and the delivery (Lavorgna et al., 2016). However, considering the increasing resistance different types of cancer show to traditional care, improving and making all these new approaches an actual therapeutic strategy would represent an important resource in cancer treatment.



### **1.3 Thyroid gland**

#### **1.3.1 Thyroid gland differentiation**

Thyroid is the main endocrine gland, responsible for the synthesis of thyroid hormones T3 and T4 that play a central role in metabolism regulation. To study this its anatomy, functioning and development, mouse is the ideal model because of the similarity of adult mouse and human thyroid anatomy and the embryonic development of the gland (Figure 3) (Fagman and Nilsson, 2010; Fernández et al., 2014).

Thyroid is mainly made of two cells types: thyroid hormones-producing follicular cells (TFCs) and calcitonin-producing parafollicular or C cells. TFCs represent the most numerous cell population in the thyroid gland and are organized in a follicular structure that holds Thyroglobulin-containing colloid. Instead, C cells are disseminated within the gland, mostly in parafollicular position (De Felice and Di Lauro, 2004). TFCs cells are characterized by the combined expression of the transcription factors Hhex, Ttf1, Pax8 and Foxe1 that are known to cooperate only in thyroid (De Felice and Di Lauro 2004; Fagman and Nilsson, 2010). Follicular cell precursors begin to express these factors at E9 of mouse embryo development, concurrently with thyroid determination beginning at E8-8.5, when the first thyroid anlage is identifiable (De Felice and Di Lauro 2004; Fagman and Nilsson, 2010; Fernández et al., 2014). At E9.5, the thyroid anlage forms the thyroid bud that at E10 begins to sever from pharyngeal floor to reach the trachea at E13.5 (De Felice and Di Lauro, 2004; Fernández et al., 2014). Functional differentiation starts at E14.5 with the expression of thyroglobulin (TG) followed the activation of the other proteins required for thyroid hormone synthesis: thyroperoxidase (TPO) and thyrotropin receptor (TSHr) (Missero et al., 1998; Fernández et al, 2014). However, T3 and T4 synthesis begins at E16.5, only after the completion of migration and follicular organization and the expression of the sodium/iodide symporter (NIS) that occur between E15 and E16 (De Felice and Di Lauro, 2004; Fernández et al. 2014).

However, whereas thyroid organogenesis is well characterized, very little is known about the first steps of thyroid commitment. Indeed, FTCs originate from endodermal cells of the midline of the foregut such as, liver, pancreas and lung primordia and it is still unclear how a subset of endoderm cells adopts a thyroid fate (De Felice and Di Lauro 2004; Fagman and Nilsson, 2010; Fagman and Amendola et al., 2011).

### **1.3.2 Thyroid cancer**

Thyroid cancer is the most common endocrine malignancy and one of the few cancers whose incidence has been increasing worldwide. Interestingly, it is described as the cancer with the greatest genetic component among not hereditary cancers. Indeed, among these ones, thyroid cancer shows the highest risk in first-degree relatives of probands.

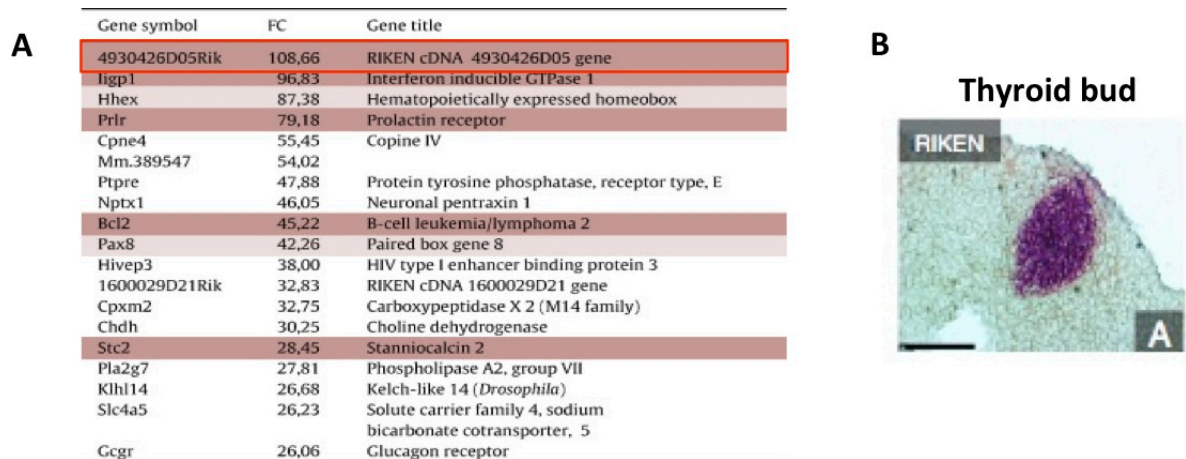
Most of thyroid cancers originate from epithelial follicular cells and are well-differentiated tumors. They usually have a good prognosis and survival rates more than 95% at 20 years (Tuttle et al. 2010). Neoplastic transformation of thyroid follicular cells can give rise to three main histotypes of thyroid carcinomas: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and anaplastic thyroid carcinoma (ATC). PTC and FTC are well-differentiated tumors and PTC is the most frequent, consisting in 85-90% of thyroid cancer cases. On the contrary, ATC is a highly aggressive undifferentiated tumor and is considered to develop from differentiated ones. It represents less of 2% of thyroid cancers, but about 40% of thyroid cancer deaths (Nikiforov et al. 2009; Katoh et al., 2015).

Genetic alterations causing thyroid cancer are well known and include point mutations and gene translocations. The first affect common oncogene such as serine/threonine kinases RAS and BRAFs, whereas chromosomal translocations occur in about 60% of PTC and 30% of FTC. Thyroid epithelium seems to have a propensity for chromosomal rearrangement (Alsina et al., 2017).

N-RAS and AKT mutations and PAX8/PPAR $\gamma$  rearrangement are most frequently found in FTC. BRAFV600E mutation and RET/PTC rearrangement are the main alterations leading to PTC, often followed by a secondary MET overexpression that promotes cell motility and invasion. TG mutations and NTRK1 rearrangements with different genes are also identified in PTC. PTEN down-regulating and TP53 up-regulating alterations characterize ATC, as well as mutations in CTNNB1 gene, involved in de-differentiation process of thyroid carcinoma and used as biomarker of tumor progression (Katoh et al., 2015; Alsina et al., 2017). Moreover, despite ATC shows a low incidence, it is lethal in the most of cases because of distant metastasis and its refractoriness against treatments (Katoh et al. 2015). Hence, it is important to identify the factors involved in thyroid carcinoma de-differentiation that could early detect tumors with poor prognosis.

## 2 Aim

Molecular mechanisms underlying early thyroid specification are still unclear. It is known that thyroid primordium expresses the transcription factor Ttf1 starting from E9.5 of mouse embryo development, but only one day later it is possible to morphologically recognize thyroid bud. So, to shed lights on early thyroid differentiation, our group performed a transcriptome analysis on E10.5 thyroid bud that picked up the transcripts enriched in this bud compared with the whole embryo. Among these, a poorly characterized gene, reported as a long non-coding RNA, emerged as the most enriched one (Figure 4) (Fagman and Amendola et al., 2011). Given these preliminary data, the main aim of my PhD work has been to characterize this lncRNA, analyzing its role in thyroid gland to understand its possible involvement in the regulation of thyroid differentiation both in normal and in neoplastic cells, and investigating its possible modes of action. The results of such work could add a rawplug leading to the final goals of understanding of what determines thyroid fate of some endodermal cells and/or which are the still unknown genetic factors involved in thyroid cancer susceptibility. Indeed, the relevance that this RNA could have in thyroid differentiation could open the way to a new approach in studying thyroid development and function.



**Figure 4. The thyroid bud most enriched gene is a poorly characterized gene.** A transcriptome analysis of thyroid bud was performed at E10.5 of mouse embryo development. The results was a list of thyroid bud enriched genes represented in A. Among these, a poorly characterized gene emerged as the most enriched one (red rectangle). Its enrichment was confirmed by in situ hybridization analysis shown in B. (Fagman and Amendola et al., 2011)

### 3 Materials and Methods

#### 3.1 RNA analysis

##### 3.1.1 Quantitative Real-Time PCR

Total RNA was isolated from cultured cells and mouse thyroids using Trizol (Sigma Aldrich) reagent according to manufacturer's specifications. Total cDNA was generated with Super Script III Reverse Transcriptase (Thermo Fisher Scientific), according to manufacturer's specifications. Real-Time PCR on total cDNA was performed with SYBR-Green mix (Bio-Rad) using gene specific oligos:

Pan Thybe1 F: GGCTCCTCTCCACTCACTTTC

Pan Thybe1 R: TCAGCTCAGCAGCGAAGTC

T1humanF: CAGCACGGACATCTGGAAGA

T1humanR: GCCTCAGCCGCCACTTTG

Pax8 F: GCCATGGCTGTGTAAGCAAGA

Pax8 R: GCTTGGAGCCCCCTATCACT

Foxe1 F: AAGCCGCCCTACAGCTACATCG

Foxe1 R: AACATGTCCTCGGCGTTGGG

TG F: CATGGAATCTAATGCCAAGAACTG

TG R: TCCCTGTGAGCTTTTGGAATG

NIS F: TCCACAGGAATCATCTGCACC

NIS R: CCACGGCCTTCATACCACC

TSHR F: TCCCTGAAAACGCATTCCA

TSHR R: GCATCCAGCTTTGTTCCATTG

TTF1 F: CTACTGCAACGGCAACCTG

TTF1 R: CCCATGCCATCATATATTCAT

Bcl2 F: GACGCGAAGTGCTATTGGTAC

Bcl2 R: CTCAGGCTGGAAGGAGAAGAT

Klhl14 F: CGATGACAGCATTTATCTAGTTGG

Klhl14 R: GCAGATTGTAGAGGACTTGTAAGGC

HK14 F: GACGCCATGAACTACCACCT

HK14i R: GCCCTCCAACCAATAACAGC

Ciclofillin F: GGATTCATGTGCCAGGGTGG  
Ciclofillin R: CACATGCTTGCCATCCAGCC  
Abelson F: TCGGACGTGTGGGCATT  
Abelson R: CGCATGAGCTCGTAGACCTTC  
Tubulin a1a F: CAACACCTTCTTCAGTGAGACAGG  
Tubulin a1a R: TCAATGATCTCCTTGCCAATGGT  
18S F: CGGCTACCACATCCAAGGAA  
18S R: GGGCCTCGAAAGAGTCCTGT

MiRNA-specific Reverse Transcriptase and Real-Time PCR were carried out using TaqMan® MicroRNA Reverse Transcription kit, TaqMan® MicroRNA Assays and TaqMAN® Universal PCR MasterMix, No AmpErase® UNG (Applied Biosystems) according to manufacturer's specification.

### **3.1.2 *In situ Hybridization***

Embryos and organs were fixed in 4% PFA, dehydrate and paraffin embedded according the following protocol:

- 4% depcPFA 4°C o.n.
- 2X Saline RT
- Saline/EtOH 96% RT
- EtOH 70% RT
- EtOH 70% 4°C o.n.
- EtOH 85% RT
- EtOH 96% RT
- EtOH 100 RT
- EtOH 100% 4°C o.n.
- 2X Xilene RT
- Xilene/paraffin 60°C
- 3X paraffin 60°C

The period of RT and 60°C steps is determined according to the size of the processed sample. Paraffin embedded samples were sliced in 7µm sections and analyzed. To perform the in situ hybridization, the sections were deparaffinized in xylene and rehydrated with EtOH 100% to EtOH 50%. After rehydration, the hybridization was performed as described in Fagman and

Amendola et al., 2011, using specific probes for Thybe1 and Klhl14 amplified from adult mouse thyroid cDNA using the following oligos:

Thybe1 sp6: GGCTGAACAGGAAGGGACCCCT

Thybe1 T7: CAGATCACAGCTAAGAAAAAAGC

K14 F: GAGGATACAGCTGGAGTATGGG

K14 R: GAGCTGAAGAGCAATAGGGTGT

### **3.2 Transcripts mapping**

cDNA from adult mouse thyroid and FRTL-5 were amplified with the oligos representing the ends obtained through RACE experiments and the 5' oligo included the upstream CAGE site reported in UCSC:

Thybe1 CAGE: CGCGTACTGCATGCGGGTCTCA

Thybe1 5'RACE: GAGAGAGGAACAACAATCAAGGC

Thybe1 3' RACE: GGGGATTAGAGTTTATTTTTGTCATCTC

The fragments obtained were blasted against Mouse and Rat genomes reported in NCBI and ENSEMBL.

### **3.3 Cell culture and transfection**

FRTL-5, ERRASV12FRTL-5 and RASV12FRTL-5 were grown in Coon's modified F12 medium (Euroclone) supplemented with 5% calf serum (Hyclone Thermo Fisher Scientific) and six hormones as described in De Vita et al. (2005). Locked nucleic acid oligos, miRNA mimics and plasmid DNA were transfected using Lipofectamine 2000® (Thermo Fisher Scientific) according with manufacturer's specifications. Tamoxifene, MAPK and PI3K inhibitors (U06126 (Biomol BPS-27012) and LY294002) were added to culture medium at 0.4 nM, 0.25nM and 0.75 nM respectively.

LNA panTb1 was designed through Exiqon tool that generated the following sequence and has ready negative controls:

LNA panTb1: AAGTGAGTGGAGAGGA

LNA negative control: AACACGTCTATACGC

### **3.4 Protein analysis**

#### **3.4.1 Protein Extraction and Western Blot**

Total proteins were extracted using a lysis buffer made of NaCl 150mM, Tris HCl 50mM, MgCl<sub>2</sub> 5mM, Na deoxycolate 0.5 %, SDS 0.1%, Triton X-100 1%. To this buffer were added DTT 0.1 mM, PMSF 0.5 mM, proteinase inhibitor cocktail (Sigma Aldrich P8340) and phosphatase inhibitor (Sigma Aldrich P0044). 20µg were loaded for western blot analysis. Immunoblots were incubated with the following antibodies: anti-Pax8 (home-made), anti-Bcl2 (Santa Cruz 7382), anti-pAKT (cell signaling 9275), anti pERK (cell signaling 9101), anti-AKT (cell signaling 9272), anti-ERK (cell signaling 9102) and anti-GAPDH (Immuno Chemical G4-C5-N).

#### **3.4.2 Immunohistochemistry**

7µm sections were obtained, deparaffinized and rehydrated as described for ISH. The sections were permeabilized with 5' PBS-0.2% triton, washed 2X 5' PBS and they underwent unmasking treatment in citrate buffer (0.01M pH6) 15' in the microwave. Endogenous peroxidases were then saturated with methanol and 1.5% oxygen peroxide and tissues permeabilized with 5' PBS-0.2% triton, washed 2X 5' PBS and blocked in blocking solution (5% Normal goat serum (Vector Laboratories S-1000), 3% BSA, 20mM MgCl<sub>2</sub>, 0.3% tween20 in PBS) 1h at room temperature. Primary antibody (α-klhl14 Abcam 49353) was used 1:200 in blocking solution overnight at 4°C. The sections then underwent to the following protocol:

5' PBS-0.2% triton, 2X 5' PBS, 1h secondary antibody (biotinilated α-rabbit IgG (H+L) Vector Laboratories BA-1000) 1:200 in blocking solution at room temperature, 5' PBS-0.2% triton, 2X 5' PBS, 30' ABC (Vector Laboratories SK-4000) RT, 5' PBS-0.2% triton, 3X 5' PBS, 2' DAB substrate (Vector Laboratories SK-4100).

The samples were then de-hydrated as described for the inclusion process and cover with cover glasses using Eukitt mounting solution (Eukitt<sup>®</sup> Quick-hardening mounting medium, Sigma-Aldrich 03989).

### **3.5 Plasmids**

Specific primers were used to amplify mouse Thybe1 Rik 04 from adult mouse thyroid:

Thybe1 5'RACE: GAGAGAGGAACAACAATCAAGGC

Thybe1 3' RACE: GGGGATTAGAGTTTATTTTGTTCATCTC

KpnI and XbaI sites, at 5' and 3' end, respectively, were included in these primers. The amplified product was subcloned into the eukaryotic expression vector pCEFL

### **3.6 Mice treatment**

Animals were kept in an animal house under controlled conditions of temperature, humidity, and light and were supplied with standard or implemented food and water ad libitum. Tg-rtTA-TetO-BRafV600E transgenic mice were fed with a 25000mg/kg Doxycycline supplemented fodder for one week. TetO-BRafV600E and Tg-rtTA transgenic mice arrived from Fagin's group in New York for a scientific collaboration and were crossed to obtain the double transgenic mice Tg-rtTA; TetO-BRafV600E to treat. All animal experiments were performed in accordance with regulations and guidelines of Italy and the European Union and were approved by the local ethical committee.



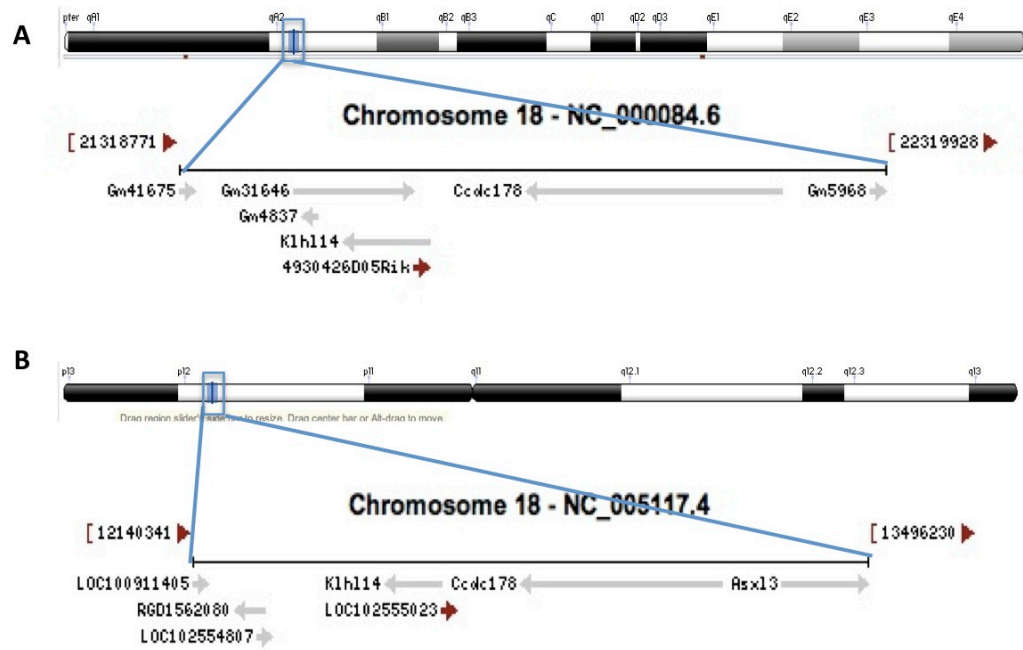
## 4 Results

### 4.1 *Thybe1 gene is expressed in different transcripts*

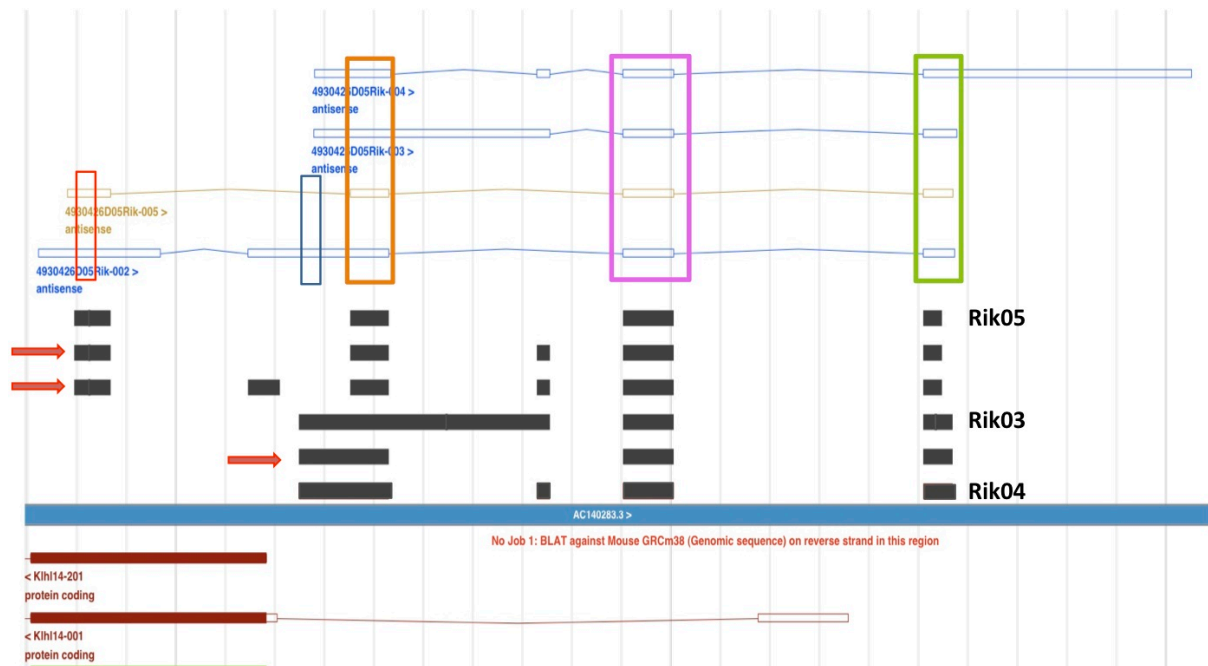
Thybe1 is a poorly characterized gene reported in genomic databases as long non-coding RNA. We looked at mouse and rat Thybe1 locus in several databases such as NCBI, UCSC and Ensembl. In mouse it is named 4930426D05Rik and maps to 18qA2 (Figure 5A), while in rat it is named LOC102555023 and maps to 18p12 (Figure 5B). Interestingly, in both genomes Thybe1 partially overlaps with the protein-coding gene Klhl14 in a head-to-head antisense arrangement (Figure 5A,B). Such overlap with Klhl14 is conserved in a wide range of vertebrate genomes, including humans (data not shown).

It is worth noting that different transcripts are reported for this gene in both mouse and rat. As shown in Figure 2A, mouse Thybe1 presents four transcripts called as 4930426D05Rik 01, 02, 03 and 04. These transcripts differ for the transcription start site and/or the exons, mainly at the 5' moiety, whereas all except Rik04 share three regions (Figure 6 orange, pink and green rectangles). As we were interested in Thybe1 function in thyroid, we decided to characterize which of the reported transcripts were expressed in this gland. To this purpose we amplified and sequenced several transcripts from adult mouse thyroid cDNA. To map 5' and 3' ends of thyroid isoforms we performed RACE experiments (data not shown) that identified a polyadenylated 3' end (Figure 6 green rectangle) but did not give unambiguous information about 5' end. For this reason we looked for annotated CAGE sites in UCSC database. Then starting from RACE identified ends and the most upstream CAGE site reported, we amplified and sequenced several fragments. Through this analysis, we revealed all the transcripts reported, except Rik02, exist and are expressed in thyroid. Moreover, we identified three differently spliced transcripts that have not been previously annotated (Figure 6).

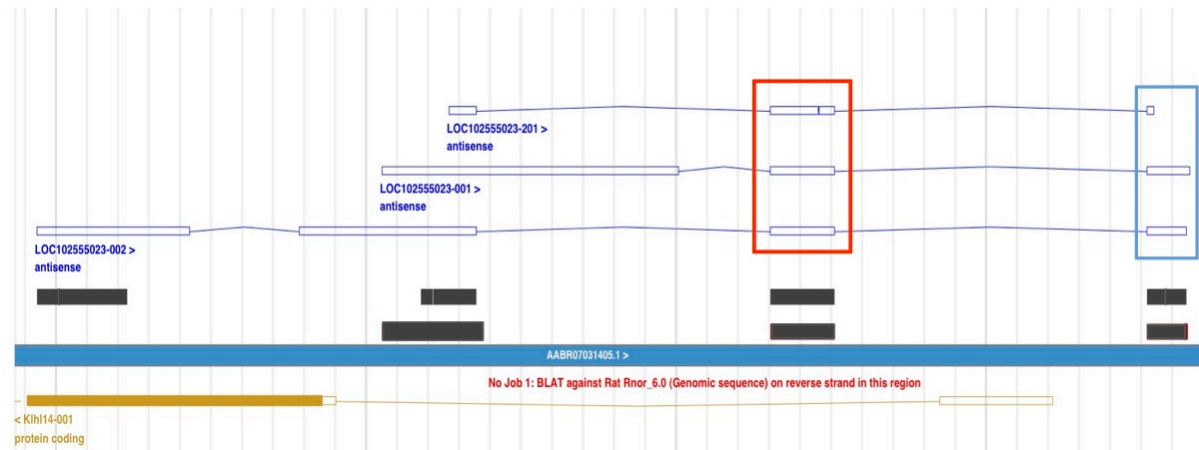
The same analysis was performed on FRTL-5 rat thyroid follicular cells cDNA, to characterize rat Thybe1 transcripts. Two different transcripts are currently reported for rat Thybe1 (Figure 7A), with different 5' ends and exon splicing, sharing the last two exons (Figure 3 red and blue rectangles). Starting from the reported ends we amplified and sequenced two transcripts that do not correspond to the reported ones (Figure 7). We will refer to these transcripts as long and short Thybe1. These results reveal that Thybe1 is expressed in mouse and rat thyroid with multiple transcripts, some of which are novel isoforms not reported in genomic databases.



**Figure 5. Thybe1 maps on chromosome 18 both in mouse and in rat.** NCBI Thybe1 locus representation shows the long non-coding gene, indicated by the red arrow, is situated on chromosome 18qA2 in mouse (A) and on chromosome 18p12 in rat (B), in the regions pointed out by blue stripe. In both species Thybe1 is located on the plus strand and shows a partially overlapping head-to-head arrangement with the protein-coding gene Khlh14 on the opposite strand



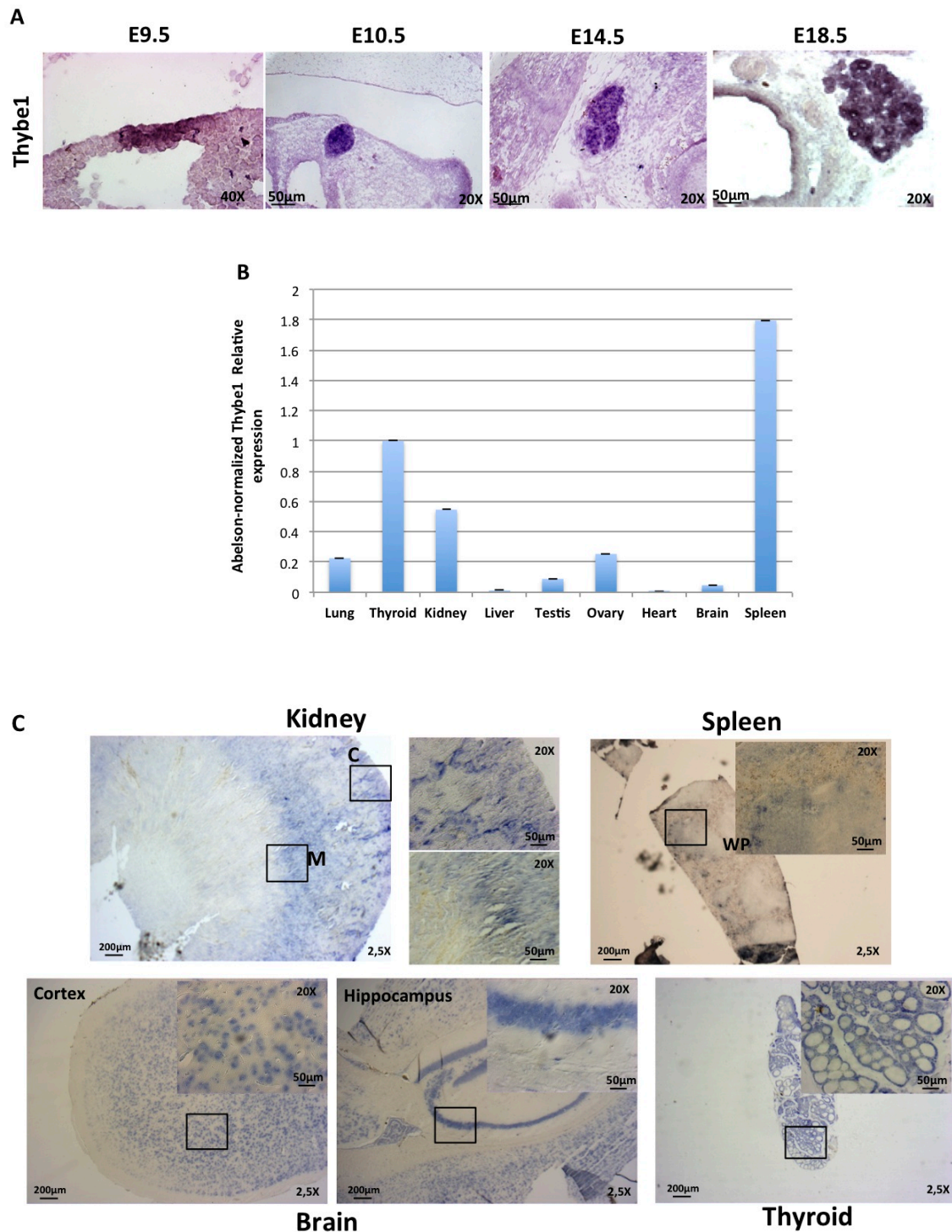
**Figure 6. Mouse Thybe1 has different transcripts.** As reported in the Ensembl representation, four different transcripts are reported for mouse Thybe1 that differ for the transcription start site and are differentially spliced, but share three regions indicated by orange, pink and green rectangles. Starting from CAGE site (red rectangle) and 5' and 3' ends identified by RACE (blue and green rectangles), we amplified several transcripts from adult mouse thyroid cDNA. Their sequencing revealed that mouse thyroid expresses different transcripts: the reported rik03, rik04 and rik05, but not rik02 and other three not annotated isoforms, indicated by red arrows.



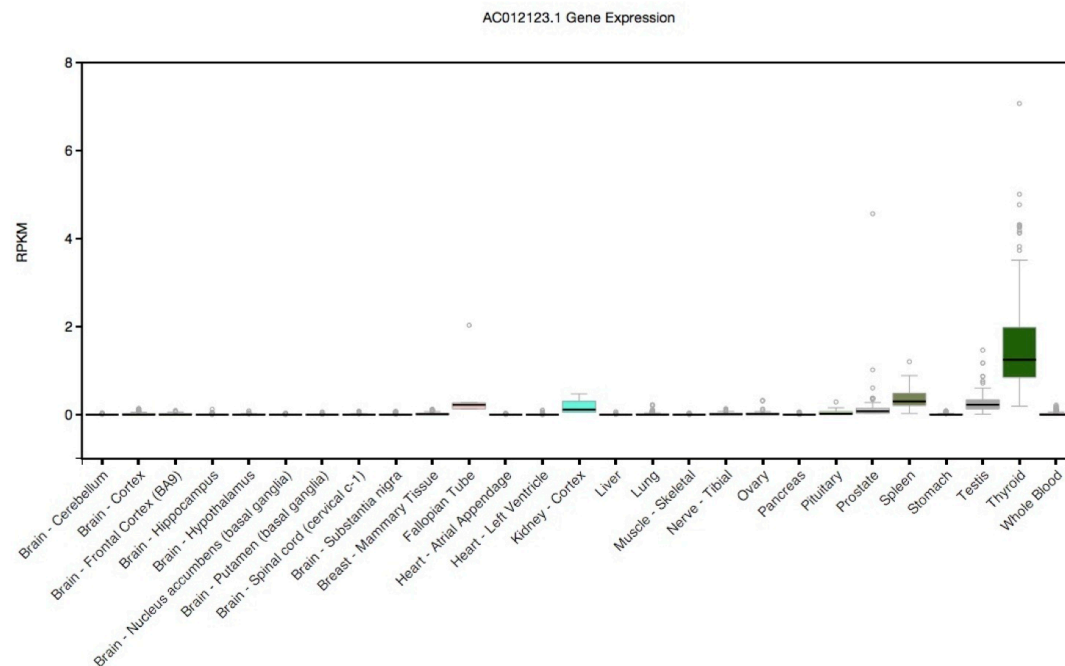
**Figure 7. Rat Thybe1 has different transcripts.** Rat Thybe1 presents three transcripts with different transcriptional start site and exon splicing but with shared last two exons (red and blue rectangles), except for transcript 201. Amplified and sequenced fragments, from rat thyroid follicular cells FRTL-5 cDNA, were blasted to rat genome. The two transcripts identified in FRTL-5 do not correspond to anyone of the annotated ones and differ for 5' regions, preserving the conserved region.

## **4.2 *Thybe1* is expressed in thyroid from embryo to adult life.**

Since *Thybe1* was found out looking for genes enriched in thyroid bud at E10.5 of mouse embryo development, we assayed its expression from this stage forwards in thyroid organogenesis and differentiation. We performed in situ hybridization (ISH) at E9.5, E10.5, E14.5 and E18.5 (Figure 8A) showing that *Thybe1* is already expressed at E9.5, the very early stage of thyroid specification and its thyroid expression is maintained until later stages of thyroid development. Given this evidence, we wondered if its strong thyroid expression is also retained in adult life. To answer this question, we evaluated *Thybe1* presence in mouse adult tissues, through qRT PCR (Figure 8B) and in situ hybridization (Figure 8C) analyses. QRT PCR gave us an idea of *Thybe1* dealing out among adult tissues, showing that it is expressed in several organs such as kidney, spleen, testis and above all, thyroid gland. However, qRT PCR considers the whole organ, overlooking the differences between different types of cells that constitute that organ. This technical limit could lead to underestimating *Thybe1* presence in given cell types. In situ hybridization, instead, let us to clearly understand the lncRNA distribution in specific cellular types. ISH performed on adult mouse organs revealed that *Thybe1* is expressed in the cortex of kidney and in the white pulp of the spleen. With respect to the brain, its expression is restricted mostly in the hippocampus and the cortex, whereas in thyroid is highly expressed in follicular cells (Figure 8C). Then we looked for data about *Thybe1* human expression profile. Gtex annotations in UCSC database report that the lncRNA is expressed in kidney, spleen, as observed in mouse, and that its enrichment in thyroid is confirmed also in humans (Figure 9) ([https://genome.ucsc.edu/cgi-bin/hgGene?hgg\\_gene=uc060ohv.1&hgg\\_prot=ENST00000426194.1&hgg\\_chrom=chr18&hgg\\_start=32769794&hgg\\_end=32774413&hgg\\_type=knownGene&db=hg38&hgsid=577587793\\_hj7rmd9LRdwjB5zqKr2VTfxokw0d](https://genome.ucsc.edu/cgi-bin/hgGene?hgg_gene=uc060ohv.1&hgg_prot=ENST00000426194.1&hgg_chrom=chr18&hgg_start=32769794&hgg_end=32774413&hgg_type=knownGene&db=hg38&hgsid=577587793_hj7rmd9LRdwjB5zqKr2VTfxokw0d) ).



**Figure 8.** Thybe1 is expressed in thyroid from embryo to adult life. Thybe1 in situ hybridization (A) shows that Thybe1 is strongly expressed in thyroid bud from E9.5 of mouse embryo development to later prenatal stage. Quantitative real time PCR (B) on adult mouse tissues reveal that Thybe1 is expressed in several tissues, in particular in kidney and spleen, showing an enrichment mainly in thyroid gland. In situ hybridization (C) confirms these data, revealing that Thybe1 is expressed in specific areas. In kidney Thybe1 is expressed in cortex. In spleen the lncRNA expression is restricted to the white pulp (WP); whereas in brain its expression is prevailing in cortex and hippocampus. In thyroid Thybe1 shows a strong expression in follicular cells.



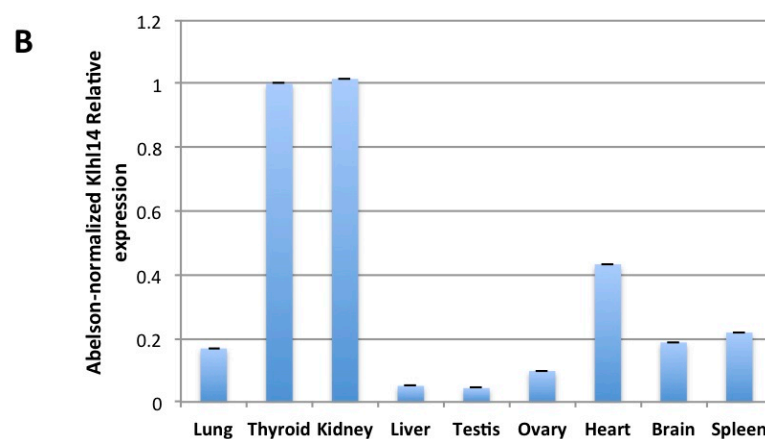
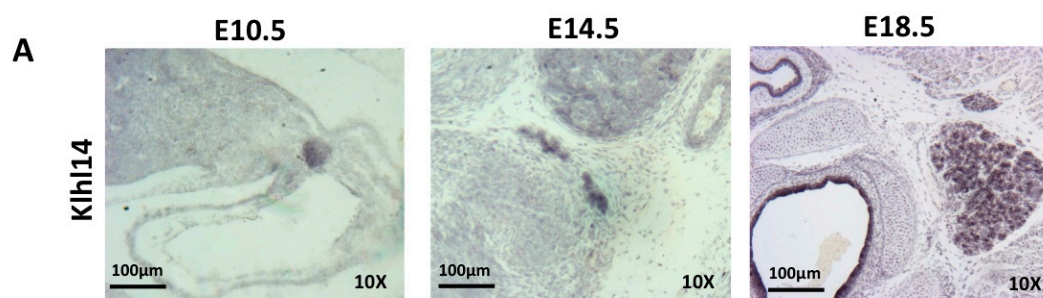
**Figure 9. Thybe1 expression profile is conserved in human.** Bioinformatic analysis from Gtex RNA-seq data shows that human Thybe1 is expressed in kidney, spleen, testis, but it is primarily expressed in thyroid ([https://genome.ucsc.edu/cgi-bin/hgGene?hgg\\_gene=uc060ohv1&hgg\\_prot=ENST00000426194.1&hgg\\_chrom=chr18&hgg\\_start=32769794&hgg\\_end=32774413&hgg\\_type=knownGene&db=hg38&hgside=577434493\\_lhpdLNxXhHdz9vkXi0a46pk2afPA](https://genome.ucsc.edu/cgi-bin/hgGene?hgg_gene=uc060ohv1&hgg_prot=ENST00000426194.1&hgg_chrom=chr18&hgg_start=32769794&hgg_end=32774413&hgg_type=knownGene&db=hg38&hgside=577434493_lhpdLNxXhHdz9vkXi0a46pk2afPA)).

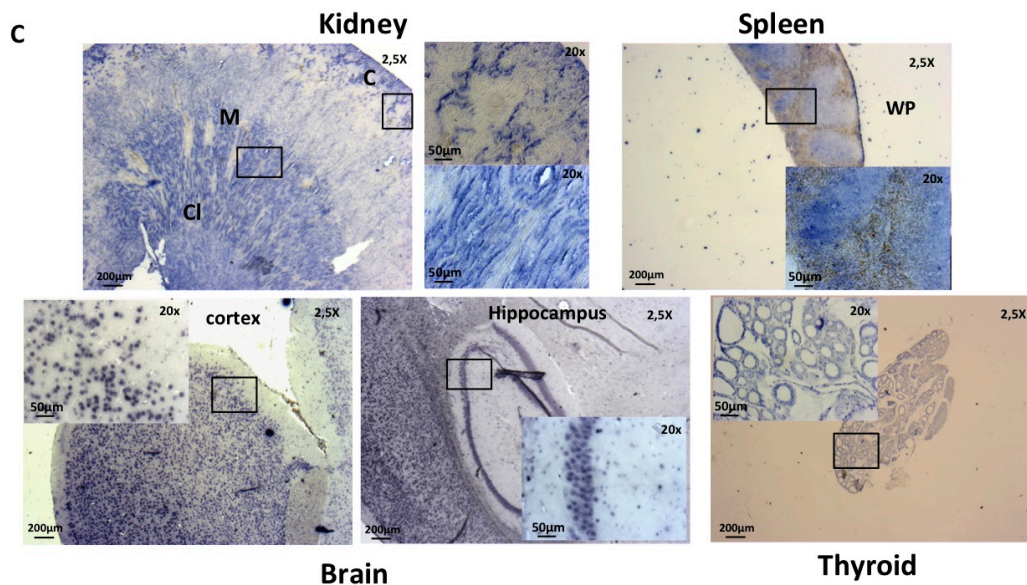
#### 4.2.1 Thybe1 overlapping gene Klhl14 shows a similar expression trend

An interesting feature of Thybe1 locus is its head-to-head overlapping arrangement with the protein-coding gene Klhl14 on the opposite strand (Figure 5). This arrangement allows to classify this lncRNA as NAT (Natural Antisense Transcript). NATs are described to be able to positively or negatively regulate the associated protein-coding gene (Zucchelli et al, 2015), leading to either a comparable or an opposite expression profile between the two genes (Wilsuz et al., 2009; Scheuermann and Boyer, 2013; Boque-Sastre et al., 2015). For this reason we investigated Klhl14 expression profile and compared it to that of Thybe1. Since Klhl14 resulted also enriched in thyroid bud at E10.5 (Fagman and Amendola et al., 2011), we decided to follow its expression during thyroid development. We performed an IHC on mouse embryos from E10.5 forward, observing Klhl14 thyroid expression is maintained during embryo development (Figure 10A), as already observed for Thybe1 (Figure 8A). Then we evaluated Klhl14 levels in adult mouse tissues through qRT PCR (Figure 10B), noting that it is expressed in several adult tissues such as kidney, heart, brain, by also retaining a strong thyroid expression. In situ hybridization on (Figure 10C) reveals that Klhl14 RNA is

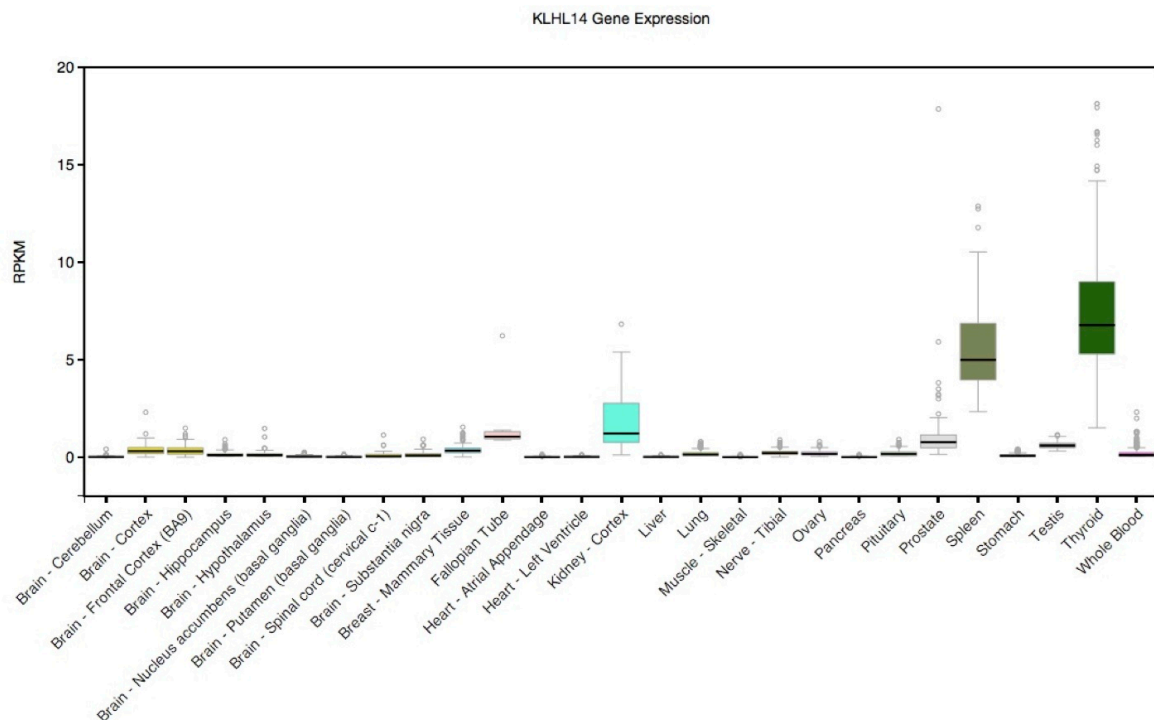


considerably expressed in thyroid follicular cells and it is located in kidney cortex, medulla and calyces, in the white pulp of the spleen and in the hippocampus and cortex in the brain , similarly to what described for Thyb1 (Figure 8C). We also looked for this protein coding gene data in human gene expression databases. Gtex annotations, from UCSC database, reporting Klhl14 as mainly expressed in kidney, brain and thyroid (Figure11)([https://genome.ucsc.edu/cgi-bin/hgc?hgsid=577587793\\_hj7rmd9LRdwjB5zqKr2VTfxokw0d&c=chr18&l=32672670&r=32773062&o=32672670&t=32773062&g=gtexGene&i=KLHL14](https://genome.ucsc.edu/cgi-bin/hgc?hgsid=577587793_hj7rmd9LRdwjB5zqKr2VTfxokw0d&c=chr18&l=32672670&r=32773062&o=32672670&t=32773062&g=gtexGene&i=KLHL14)). All these data show that Thyb1 and Klhl14 have a similar expression profile, so it is likely that Thyb1 could positively regulate Klhl14.





**Figure 10. Khl14 expression profile is similar to Thybe1.** Khl14 IHC(A) shows Khl14 is strongly expressed in thyroid bud from E10.5 of mouse embryo development to later prenatal stage. Quantitative real time PCR (B) on adult mouse tissues reveals that khl14 is expressed in several tissues such as kidney, lung, heart, spleen and brain, evidencing a strong thyroid expression. In situ hybridization (C) shows Khl14, as well as Thybe1, is expressed in specific cellular types. In kidney it is expressed in cortex, medulla and also calyces (CI). In spleen Khl14 is expressed in the white pulp. Brain shows khl14 enrichment in cortex and hippocampus and follicular thyroid cells expression was confirmed.

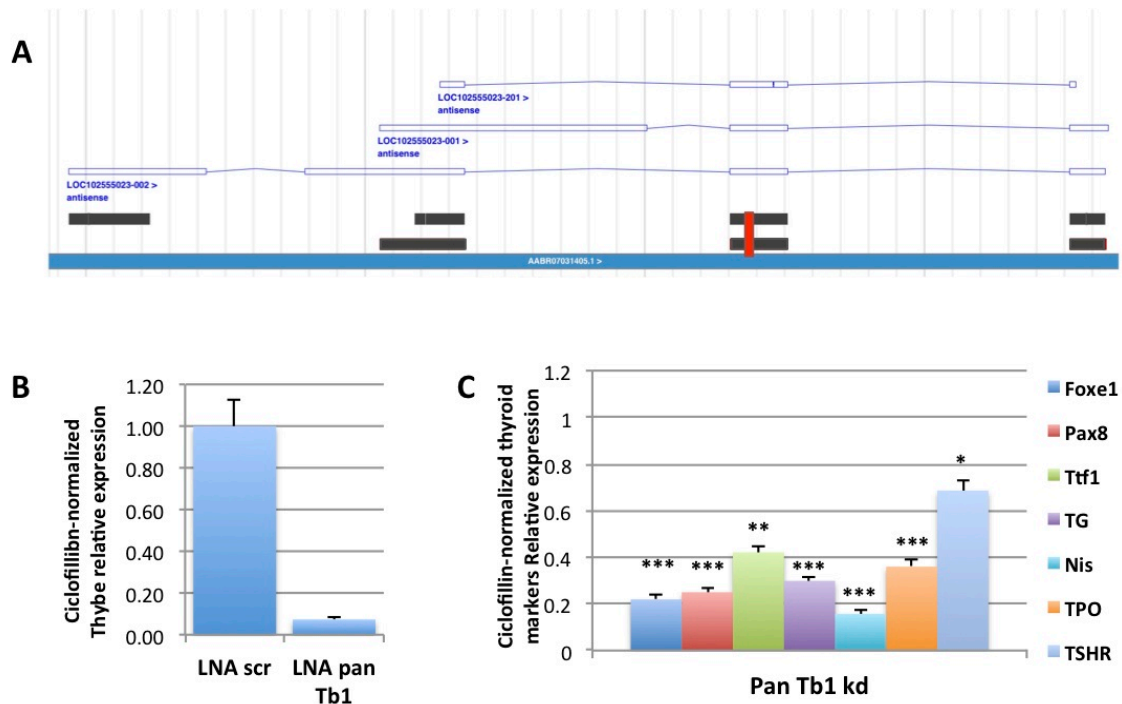


**Figure 11. Khl14 expression profile is conserved in human.** Bioinformatic analysis from Gtex RNA-seq data shows that human Khl14 is expressed in kidney and spleen but it is primarily expressed in thyroid gland ([https://genome.ucsc.edu/cgi-bin/hgchgsid=577434493\\_lhpdLNxXhHdz9vkXi0a46pk2afPA&c=chr18&l=32672670&r=32773062&o=32672670&t=32773062&g=gtexGene&i=KLHL14](https://genome.ucsc.edu/cgi-bin/hgchgsid=577434493_lhpdLNxXhHdz9vkXi0a46pk2afPA&c=chr18&l=32672670&r=32773062&o=32672670&t=32773062&g=gtexGene&i=KLHL14)).



### **4.3 *Thybe1 is required for thyroid differentiation in vitro***

To investigate if Thybe1 plays a role in thyroid differentiation we down-regulated its expression in rat thyroid follicular cells FRTL-5. FRTL-5 are adult wild type thyroid epithelial cells that express all thyroid differentiation markers (Fusco A et al., 1987; Berlingieri et al., 1988), including Thybe1, as it is shown in Figure 12B. We used locked nucleic acid oligos (LNAs) to obtain Thybe1 knockdown and then we measured thyroid marker levels by quantitative real time PCR. LNAs longRNA GapmeRs are DNA antisense oligonucleotides in which the ribose ring is “locked” by a methylene bridge. These molecules are complementary to their RNA target and when introduced into cells, they sequester their target RNA in highly stable DNA: RNA heteroduplexes, leading to RNase H-mediated target degradation. An LNA targeting the common region (LNA pan Tb1) was used to repress all Thybe1 transcripts (Figure 12A,B). Thybe1 knocking-down causes the decrease of all the analyzed thyroid differentiation markers, although at different extents. Among these, the most dramatically down-regulated are the transcription factors Foxe1 and Pax8, Thyroglobulin (TG), Sodium/Iodide Symporter (Nis) and Thyroperoxidase (TPO), while TTF-1 and TSH receptor show a lower, though statistically significant, decrease (Figure 12C). It is interesting to note that none of the down-regulated genes is located on the chromosome 18, indicating Thybe1 ability to act in trans.



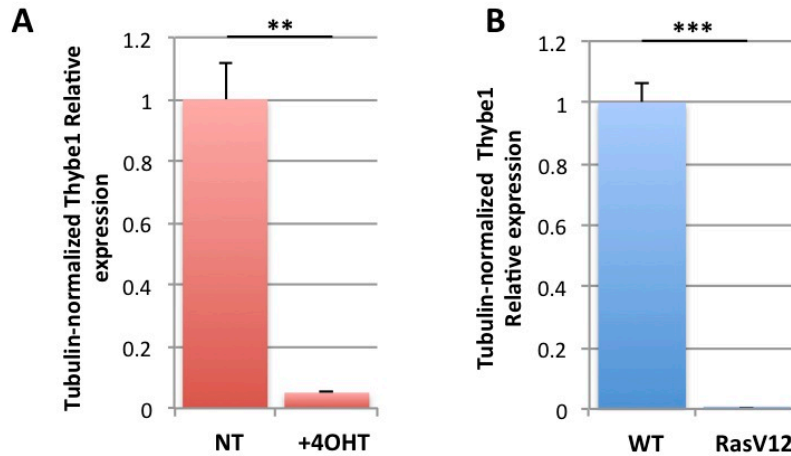
**Figure 12.** Thybe1 knockdown affects thyroid differentiation in vitro. A. Thybe1 was down-regulated in FRTL-5 transfecting 100nM of locked nucleic acid targeting the conserved region (LNA pan Tb1), in red. RNA was extracted 48h after transfection. Quantitative Real Time PCR for Thybe1 (B) and thyroid differentiation markers (C) shows Thybe1 Knocking-down leads to the down-regulation of all thyroid markers analyzed. All values are normalized to ciclofilin and expressed relatively to LNA scramble set to a value of 1. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

#### 4.4 Thybe1 is repressed in thyroid neoplastic transformation

##### 4.4.1 In vitro Ras activation suppresses Thybe1 expression

LncRNAs are acquiring an increasingly relevant role in cancer (Huarte, 2015; Lavorgna, 2016). Alterations and/or increase of lncRNAs are reported to be correlated to cancer development, some of which representing a prognostic markers of cancer progress (Gibb et al., 2011; Orfanelli et al., 2015; Xia et al., 2017). Being Thybe1 involved in thyroid differentiation, we asked if it could be also involved in thyroid cancerogenesis. To this aim we used two in vitro models of thyroid transformation with de-differentiation: ERRasV12 FRTL-5 and RasV12FRTL-5 cell lines. ERRasV12 FRTL-5 is an inducible system that expresses an oncogenic and tamoxifene (4OHT)-inducible form of Ras (ER-RasV12). These cells rapidly undergo neoplastic transformation upon 4OHT treatment. The RasV12FRTL-5,

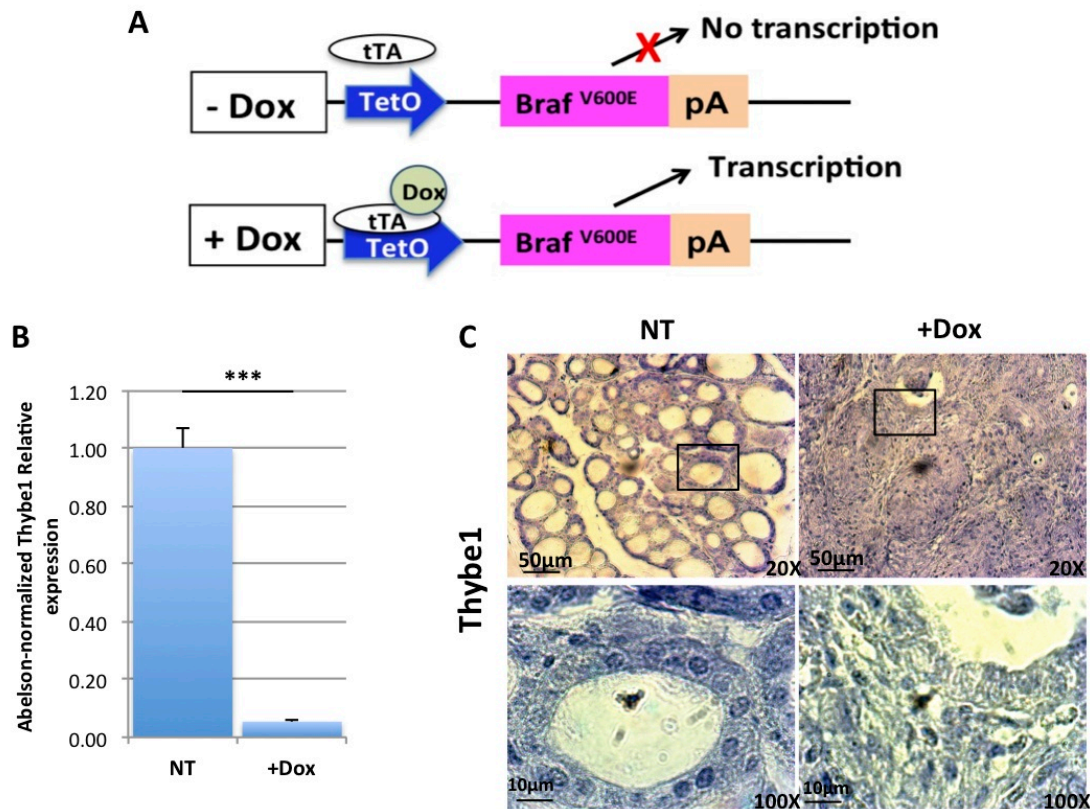
instead, express RasV12 constitutively, showing a chronically transformed phenotype (De Vita et al, 2005). We evaluated Thybe1 expression by qRT PCR in both systems, revealing that both acute and chronic Ras constitutive activation dramatically suppresses lncRNA expression compared to the controls, untreated ERRasV12FRTL-5 and WT FRTL-5, respectively (Figure 13A,B).



**Figure 13. Thybe1 is repressed by Ras activation.** Thybe1 expression was evaluated by qrt PCR analysis, in inducible ERRasV12FRTL-5 induced with 1nM 4OHT for 24h (A) and in Ras costitutively expressing FRTL-5RasV12 cells (B). The RNA expression levels are tubulin-normalized and relative to the controls (left bars). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

#### 4.4.2 *Thybe1 is repressed in BRAf-driven thyroid carcinogenesis in vivo*

To further investigate Thybe1 involvement in thyroid cancer, we exploited an inducible thyroid cancer mouse model. In this model, doxycycline (dox) administration induces thyroid specific BRAfV600E oncogene transcription (Figure 14A), leading to a poorly differentiated thyroid cancer (Chakravarty et al., 2011). Mice were treated with dox for one week and thyroid lobes were withdrawn and subjected to paraffin inclusion and RNA extraction. QRT PCR (Figure 14B) and in situ hybridization (Figure 14C) showed that Thybe1 is severely down-regulated in transformed and poorly differentiated thyroids compared to the normal ones.

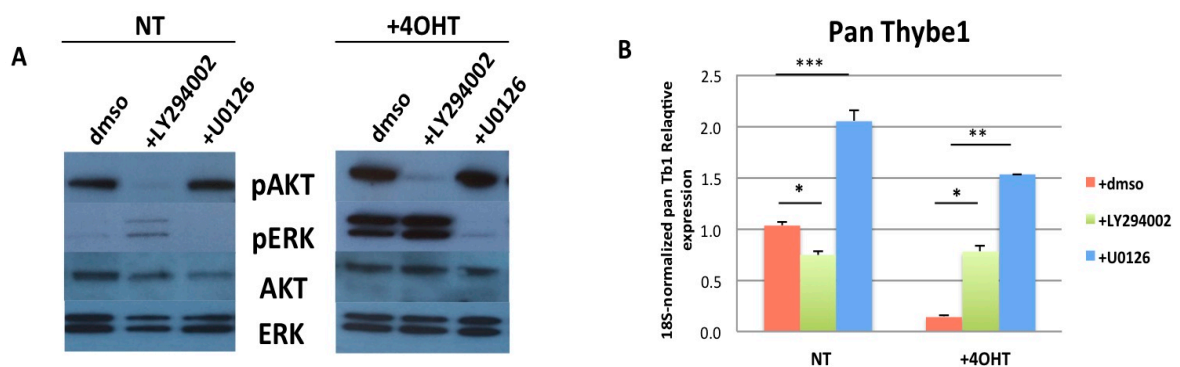


**Figure 14. Thybe1 is lost during BRAf-dependent thyroid neoplastic transformation in vivo.** An inducible thyroid cancer mouse model was tested for Thybe1 expression. A. In this model, one week of doxycycline (dox) administration induces a specifically thyroid BRAfV600E expression, causing a poorly differentiated thyroid cancer (Chakravarty et al., 2011). B. QRT pc on pools of control (NT) and dox treated thyroids (+Dox) shows a dramatic reduction of Thybe1 levels. RNA levels were normalized on Abelson and expressed relatively to controls. C. Thybe1 in situ hybridization on paraffin-embedded control and treated thyroids confirms Thybe1 down-regulation. \*\*\*,  $P < 0.001$ .

#### 4.4.3 Different signaling pathways could mediate Thybe1 repression in neoplastic transformation

The data obtained in the two experimental models of thyroid cancerogenesis show that either Ras, in the in vitro model, or BRAf, in the in vivo model, exert inhibitory effects on Thybe1 expression. We thus asked if the BRAf pathway is the only Ras-activated pathway responsible for the observed Thybe1 repression. To investigate the role of the two main Ras.-downstream pathways in down-regulating Thybe1 expression, we pharmacologically inhibited MAPK or PI3K pathways during Ras activation in vitro. To this purpose, we treated ERRasV12 FRTL-5 with tamoxifene (4OHT) together with either MEK inhibitor U0126 or PI3K inhibitor LY294002. We evaluated the phosphorylation of ERK and AKT, by western blot analysis, to

confirm MEK and PI3K inhibition (Figure 15A). Then we performed a qRT PCR analysis, observing that Thybe1 Ras-induced repression is rescued by both BRAf and PI3K inhibition, although at different extents (Figure 16B), revealing that both pathways are required, but none is sufficient per sé, for Ras-induced Thybe1 repression. Indeed, BRAf inhibition up-regulates Thybe1 in basal condition and keeps at bay its repression upon Ras activation, suggesting that MAPK pathway exerts a negative effect on Thybe1 expression. Meanwhile PI3K inhibition weakly down-regulates lncRNA in non-transformed cells, and partially rescue its suppression by Ras (Figure 15B), hinting BRAf and PI3K pathways cooperates to repress Thybe1 expression in transformed cells.

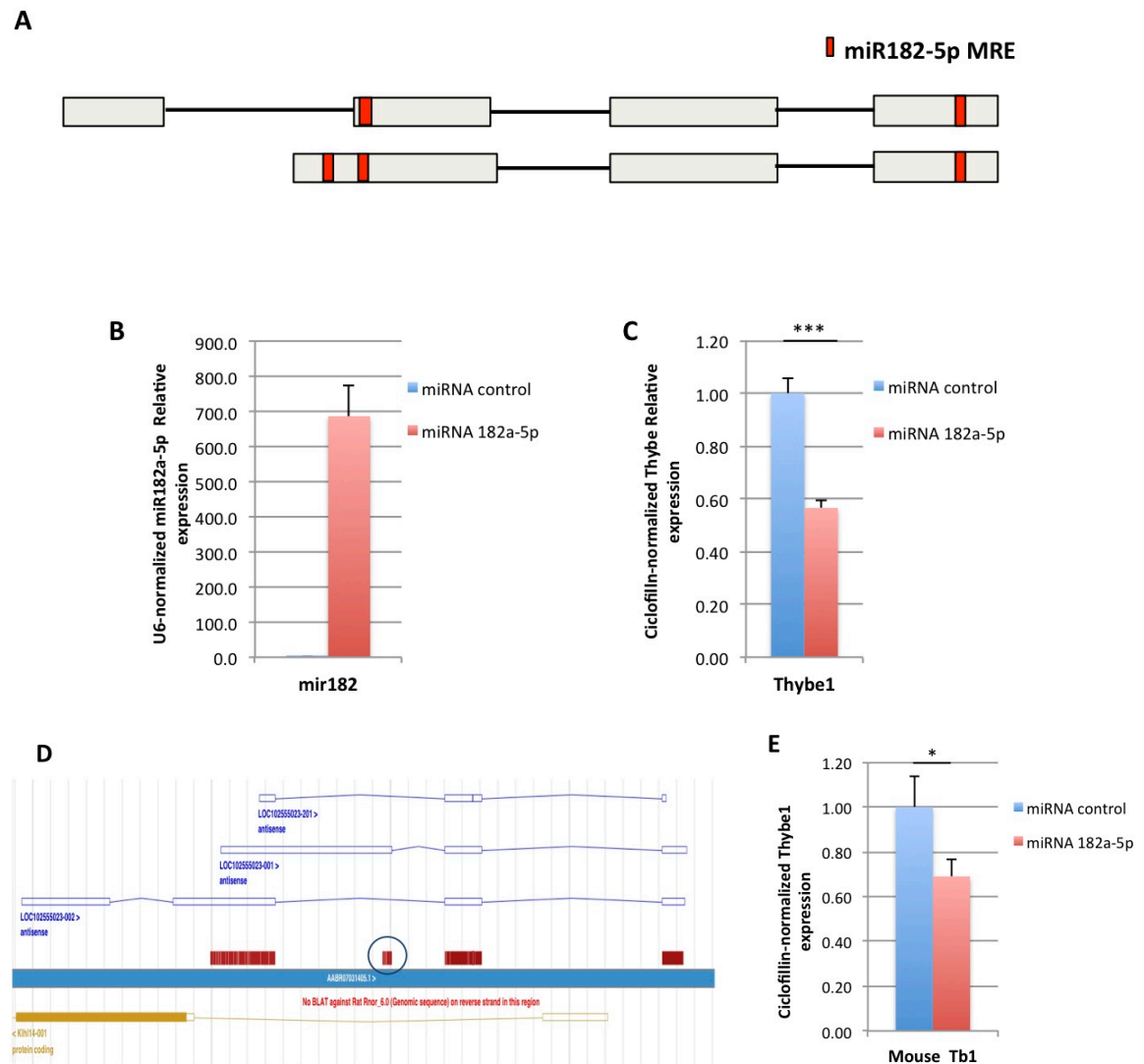


**Figure 15. Ras Thybe1 expression is rescued by PI3K BRAf pathways inhibition.** PI3K and BRAf pathways were inhibited in basal condition (NT) and after Ras activation (+4OHT), in ERRASV12 FRTL-5 using 75nM LY294002 and 25nM U0126 respectively. After 24h RNA and proteins were isolated. (A). AKT and ERK activation was checked by western blot analysis. QRT PCR were performed for pan thybe1 (B) in dms control (red bars), PI3K (green bars) and MAPK (blue bars) inhibition, revealing BRAf activity per sé is not sufficient to down-regulate Thybe1. All values are normalized to 18S and expressed relatively to dms controls. \*, P< 0.05; \*\*, P< 0.01; \*\*\*, P< 0.001.

#### 4.4.4 Thybe1 is targeted by miR182a-5p, that is up-regulated in thyroid tumorigenesis

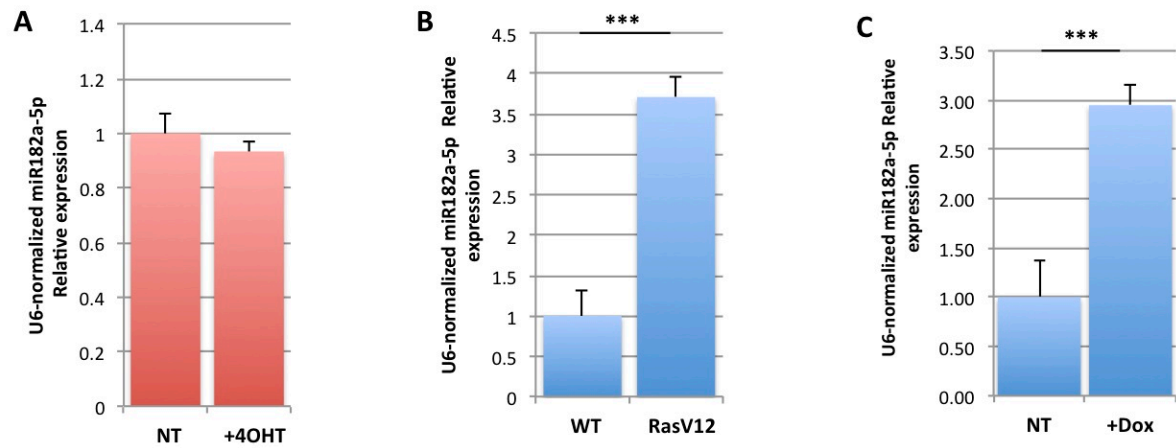
To investigate the possible mechanisms of Thybe1 repression in transformation, we asked if it could be regulated by a miRNA-mediated mechanism. Since Thybe1 is still poorly characterized, we did not find informations about the presence of miRNA-responsive elements (MRE) in its transcripts in standard miRNA targets databases. However, the RNAHybrid 2.1.2 tool predicted it contains MRE for miRNA182a-5p

(<https://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid>) (Figure 16A). Considering these indications, we decided to test Thybe1 responsiveness to this miRNA in vitro. We transfected FRTL-5 with miR182a-5p mimic and measured Thybe1 levels, observing that the expression of this miRNA (Figure 16B) leads to Thybe1 decrease (Figure 16C). To check if mir182a-5p is directly acts on Thybe1 RNA, we tested if miR182a-5p mimic is able to regulate the expression of mouse Thybe1 cDNA cloned in an expression vector. We cloned mouse Thybe1 sequence corresponding to the rat short transcript with the difference of a mouse specific 50bp exon, that allowed us to discriminate exogenous from endogenous Thybe1 RNA (Figure 16D). This transcript was cloned in a pcefl expression vector and co-transfected in FRTL-5 with the miRNA mimic. Then we specifically measured exogenous Thybe1 expression level by qrt PCR, observing that it is down-regulated by miR182a-5p overexpression (Figure 16E). MiR182a-5p is already described to correlate with different type of cancer. In particular, it was described to be up-regulated in hepatocellular carcinoma, bladder ,breast and prostate cancer (Wang at al., 2014; Wang et al 2014; Chiang et al., 2013; Hirata et al., 2012; Peng et al., 2013; Wei et al, 2015). Given the targeting of Thybe1 by miRNA182a-5p, we wondered if this miRNA is regulated also in thyroid neoplastic transformation. To answer this question we assayed miRNA levels in ERRASV12FRTL-5, RasV12FRTL-5 and in BRAf-dependent thyroid cancer mouse model, performing qRT-PCR. Interestingly we observed that miR182a-5p expression is not affected by Ras acute activation (Figure 17A), whereas it is strongly up-regulated in the same cell system by chronically active Ras (Figure 17B). Consistently, it is also strongly upregulated in undifferentiated BRAf induced thyroid cancer in vivo, (Figure 17C).



**Figure 16. Thybe1 is a miR182a-5p target.** (A) Representation of the miR182a-5p MRE on rat Thybe1 sequence from RNAHybrid 2.1.2 (<https://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid>). FRTL-5 were transfected with 5nM miR182a-5p mimic for 48h. QRT PCR for miR182a-5p (B), panTb1 (C) reveal miR182a-5p effect on endogenous Thybe1. (D) Mouse Thybe1 transcript, corresponding to short Thybe1, except for a 50bp exon (blue circle), was cloned in pcefl expression vector. (E) FRTL-5 co-transfected with 0.5 $\mu$ g of pcefl-mouse Tb1 and miR182a-5p mimics at a final concentration of 25nM. 48h after transfection, RNA was isolated and qRT PCR confirms miRNA action on Thybe1 RNA. LncRNA and miRNA levels were normalized to Ciclofillin and U6. Expression values are relative to miRNA control (left bars). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



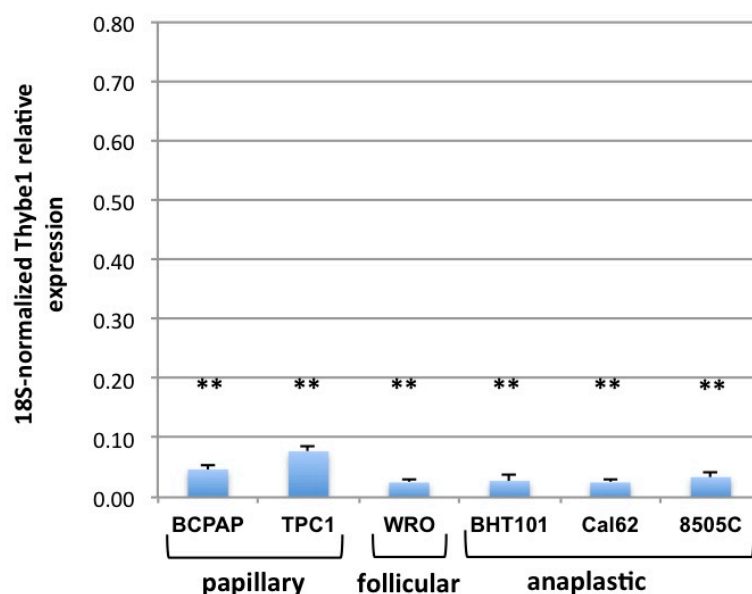


**Figure 17. miR182a-5p is up-regulated by long-term oncogene activation.** (A). QRT PCR for miR182a-5p shows a miRNA up-regulation in 24h induced ERRasV12 FRTL-5 (+4OHT), RasV12 FRTL-5 (B) and doxycycline induced BRAf-dependent thyroid cancer (+Dox) (C). Thyroid cancer induction lasted one week. MicroRNA levels were normalized to U6 and are expressed relatively to the controls (left bars). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

#### 4.4.5 *Thybe1* expression is suppressed in human thyroid cancer

Considering the previous results, we asked if *Thybe1* could be repressed also in human thyroid cancer. Neoplastic transformation of thyroid follicular cells can give different histotypes of thyroid cancer: follicular and papillary, that are well-differentiated cancers and anaplastic, that is the most undifferentiated and aggressive one (Nikiforov et al. 2009; Katoh et al., 2015). To get an idea of *Thybe1* behavior in these different human thyroid cancer variants, we analyzed human thyroid cancer cell lines that represent each of these histotypes: BCPAP and TCP1 for papillary cancer, WRO for follicular cancer and BHT101, 8505C and Cal62 for the anaplastic one (Saiselet et al., 2012). QRT PCR revealed that, compared to healthy samples, *Thybe1* is severely down-regulated in all tumor cells, without distinguishing among different histotypes, differentiation state or aggressiveness (Figure 18).

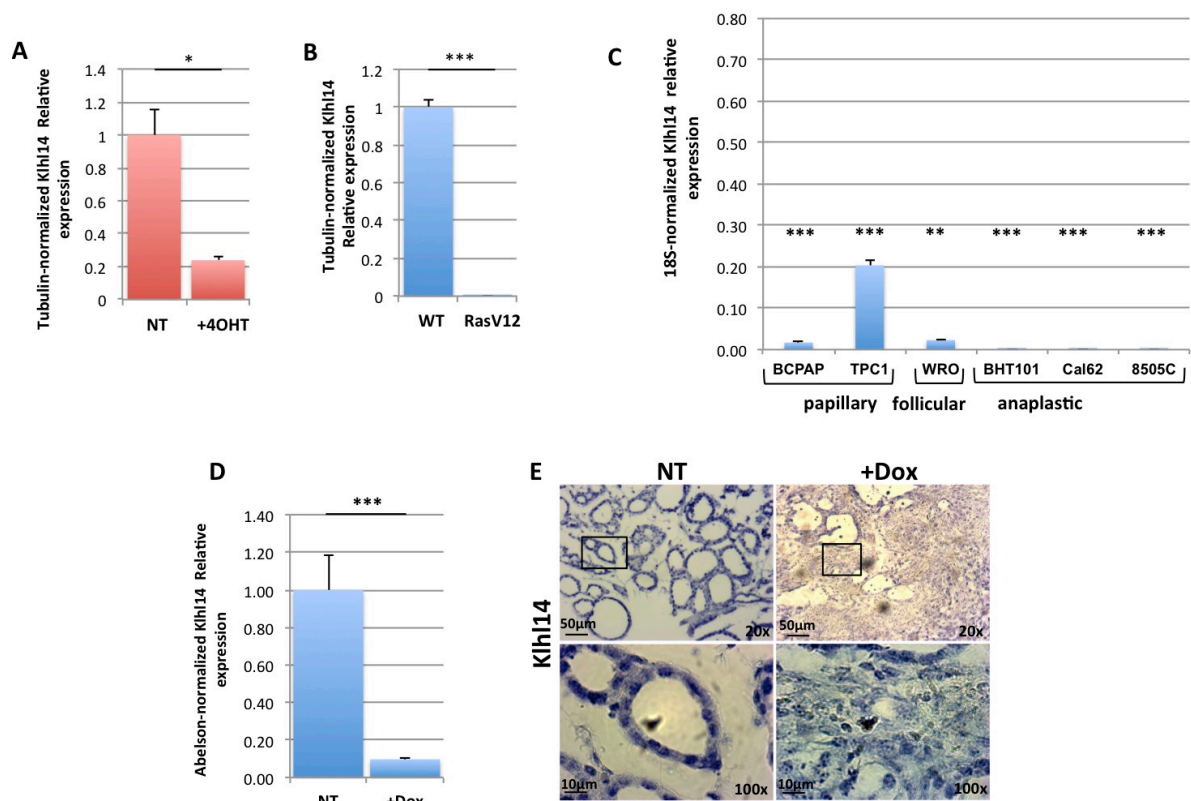




**Figure 18. Thybe1 is down regulated in human thyroid cancer.** Qrt PCR was performed to measure Thybe1 expression in papillary (BCPAP, TPC1), follicular (WRO) and anaplastic (BHT101, Cal62, 8505C) thyroid cancer cell lines, after normalization to 18S. lncRNA expression is relative to an healthy samples pool. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

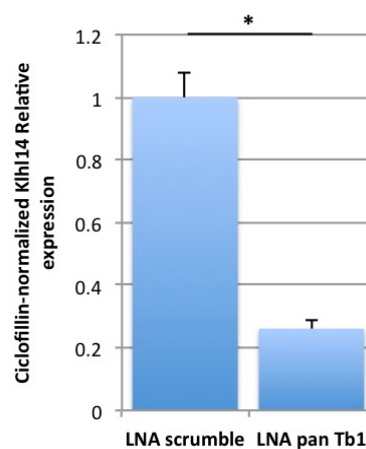
#### 4.4.6 *Klhl14 is repressed during thyroid tumorigenesis*

Considering that both Thybe1 and Klhl14 are expressed during thyroid development and they seem to be co-regulated, we wondered if also Klhl14 is involved in thyroid neoplastic transformation. To answer this question we evaluated its expression in the described models of thyroid de-differentiation and tumorigenesis. Interestingly we uncovered that, as observed for Thybe1, Klhl14 is severely repressed, in vitro, by Ras activity in ERRASV12FRTL-5 and RasV12FRTL-5 (Figure 19A,B). Besides, Klhl14 arises as down-regulated in human cancer cell lines with essentially the same trend of Thybe1 (Figure 19C). Moreover, qRT PCR (Figure 19D) and in situ hybridization analyses (Figure 19E) evidenced it is suppressed in BRAf-dependent thyroid carcinogenesis in vivo. All these data strongly correlate Klhl14 expression to Thybe1 presence suggesting its direct role in controlling the protein-coding gene expression or that the two genes undergo to the same regulatory mechanisms. To investigate in this sense we measured by qRT PCR Klhl14 expression in Thybe1 knocking-down FRTL-5. We found out that Klhl14 is down-regulated by Thybe1 knocking-down hinting that the lncRNA presence is required to preserve the protein coding expression (Figure 20)



**Figure 19. Khl14 is repressed by thyroid neoplastic transformation.** QRTPCR was performed to evaluate Khl14 levels in 24h-induced ERRasV12 FRTL-5 (A), RasV12 FRTL-5 (B). Different histotypes of human thyroid cancers (C) and in doxycycline induced BRAF-dependent mouse thyroid cancer (+ Dox)(D). RNA levels are normalized on Tubulin, 18s and Abelson respectively. In A, B ,C and D, RNA levels are expressed relatively to controls set to a value of 1(left bars); in C they are relative to a pool of healthy samples set to a value of 1 (not shown). (E) Khl14 In situ hybridization on paraffin embedded sections was performed on dox treated thyroids. Thyroid cancer induction lasted one week. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Figure 20. Khl14 negatively regulated by Thybe1 repression.** FRTL-5 upon Thybe1 interfering, with 100nM LNA pan Tb1 (right bar), shows a down-regulation of Khl14 expression. RNA was isolated 48h after transfection. mRNA levels are normalized to ciclofillin and relative to the LNA control. \*,  $P < 0.05$



## 5 Discussion

In this thesis we report the structural and functional study of a novel gene for a long noncoding RNA. The discovery of such gene stems from previous studies of our group aimed at investigating the molecular bases of thyroid specification. A screening for genes specifically characterizing thyroid development and commitment identified a non-coding gene as the most enriched in E10.5 mouse thyroid bud. We named this poorly characterized gene Thybe1 (Thyroid bud enriched 1). Thybe1 overlaps, in a head-to head arrangement, with a coding gene, Klhl14, also enriched in thyroid bud. The lncRNA chromosome location and its overlapping with Klhl14 are conserved in mouse, rat and other species, among which human. The evolutionary conservation of such genomic organization strongly suggests that it is critical for the function of this pair of genes. To investigate Thybe1 expression profile we consulted databases for human tissues and performed an experimental observation for mouse tissues. The two approaches revealed that Thybe1 is expressed in many tissues, even if at low level, similarly to most of lncRNA (Cabili et al., 2011; Morlando et al., 2014; Marques and Ponting, 2009), but more important, it is expressed mainly in thyroid, from very early stage of thyroid specification, during embryo development, to adult life, hinting that Thybe1 presence is important for gland development and function. Moreover, sequencing of several fragments, amplified from adult mouse thyroid and rat thyroid follicular cells, revealed that in addition to those already reported in genomic databases, novel splicing transcripts of Thybe1 exist and are expressed in thyroid, suggesting that this gene plays a relevant role in this gland, or better still, could play more than one role in thyroid development and function. To understand its functional role, we knocked-down Thybe1 in normal thyroid cells, obtaining the repression of most differentiation markers such as the transcription factors Foxe1, Pax8, Ttf1, Thyroglobulin, Sodium-iodide/symporter (Nis) and Thyroid peroxidase (TPO). Such widespread effect on differentiation-related genes, could be either a direct effect on each of the regulated genes, or more likely could be the indirect consequence of the deregulation of one or more transcription factors, whose reduction, in turn, leads to the loss of the other markers. However, these data highlight that Thybe1 is able to act *in trans* on its targets, since none of the down-regulated genes is situated near to or on the same chromosome of lncRNA locus.

lncRNAs employ several different molecular mechanisms to activate or repress neighbors and/or distant genes (Wilsuz et al., 2009; Guttman et al., 2012; Scheuermann and Boyer, 2013; Marchese and Huarte, 2014; Fatica and Bozzoni, 2014) that could be involved in differ

biological events. In particular, an increasing number of studies have outlined the role of several lncRNAs in cancer (Huarte, 2015; Lavorgna, 2016). Considering the role of Thyb1 in thyroid differentiation, we tested if it could be involved also in loss of differentiation that occurs in thyroid cancer development. We thus decided to investigate if Thyb1 is regulated by oncogenic transformation of thyroid cells. To this purpose, we firstly measured its expression levels in chronic as well as inducible cellular models of Ras-induced thyroid transformation (De Vita et al., 2005). Interestingly we observed that Thyb1 expression is dramatically reduced by both acute and chronic oncogenic activation. These results were also confirmed *in vivo* by using a Doxycycline-inducible thyroid cancer mouse model, where BRAFV600E activation induces a poorly differentiated thyroid cancer (Chakravarty et al., 2011). We discovered that, in such cancer, Thyb1 levels are considerably lower than in normal thyroid, confirming that this lncRNA is strongly repressed during transformation and strictly linked to the expression of differentiation-specific genes. Chemical inhibition of both BRAF/MAPK and PI3K pathways in cellular models partially rescued the inhibition of Thyb1 by Ras, showing that activation of BRAF is required but not sufficient per sé for Thyb1 inhibition. However, during BRAF-dependent thyroid carcinogenesis *in vivo*, many other alterations occur that could lead to the lncRNA repression.

To test if the observed Thyb1 repression could be mediated by a miRNA-mediated mechanism, we looked for MREs on Thyb1 sequence, finding miRNA182a-5p responsive elements (<https://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid>). For this reasons, we validated *in vitro* Thyb1 as a target of this miRNA, transfecting miR182-5p mimics in FRTL-5, finding out that it is repressed by the miRNA increase. Moreover, interestingly, in the described *in vitro* system, miR182a-5p expression is strongly upregulated by chronic oncogenic Ras activation, while it is unaffected at early stages of Ras acute induction. miR-182a-5p was also highly up-regulated in the BRAF induced thyroid cancer *in vivo*, thus supporting the data obtained on *in vitro* system. This miRNA is already known to be up-regulated in other type of cancer, although these studies refer to human cancers and no data are available on its expression during cancer progression. Taken together, our data suggest that upregulation of miR182a-5p is not an early event in oncogenic transformation of thyroid cells, yet its increase can occur in later stages of thyroid cancer development. Thus, the early repression of Thyb1 by Ras is likely to be mediated by other mechanisms, while miR182a-5p could be involved in the long-term Thyb1 inhibition observed both in cultured cells and in tumors induced in the mouse model of thyroid cancer.

Our observations could also lead to speculate that while miR182-increase could be necessary

to maintain Thybe1 down-regulated in late stages of cancer development, Thybe1 early repression could release a possible sponge effect on miR182a-5p, rendering it available to target other RNAs involved in the tumor progression process. To understand what really happens, it would be useful to identify Thybe1 promoter and test its activity in transformed and/or tumor conditions, and to test the expression of other targets of this miRNA in transformed thyroid cells.

To verify if Thybe1 was also repressed in human thyroid cancer, we measured its levels in human follicular, papillary and anaplastic cancer cell lines, uncovering that Thybe1 is strongly repressed in all these cells compared to normal samples. Taken together, these data demonstrate that Thybe1 is implicated in thyroid cancerogenesis, without differences between different histotypes and it is probably lost at early stages of thyroid neoplastic transformation. Thybe1 has another interesting feature that is the overlap with the protein-coding gene Klhl14, also enriched in thyroid bud. Their head-to-head arrangement allowed us to classify Thybe1 as a Natural Antisense Transcript (NAT). NATs are known to regulate the expression of the corresponding protein-coding gene in positive or negative way (Wilsuz et al. 2009; Ørom et al., 2010; Boque-Sastre et al., 2015). Given this relationship, we investigated Klhl14-Thybe1 expression correlation. We analyzed Klhl14 expression profile observing that it is expressed during thyroid bud development from E10.5 to mouse adult gland, similarly to the lncRNA. Moreover it shows a tissue expression trend similar to that of Thybe1, being mostly expressed in thyroid. Interestingly we also observed that, like Thybe1, Klhl14 is strongly down-regulated by thyroid transformation and tumorigenesis *in vitro* and *in vivo*. These data suggest that Thybe1 and Klhl14 are regulated by the same factors and/or Thybe1 positively regulates Klhl14 expression. The down-regulation of Klhl14 upon Thybe1 knocking-down, observed in cultured cells, supports the second hypothesis, also suggesting that it is not Thybe1 transcription to induce Klhl14 expression. NATs are described to positively regulate the overlapping protein-coding gene by recruiting the transcription complex to their own promoter and so promoting also the transcription on the opposite strand. Others lncRNAs act as so called “scaffold RNAs”, binding and stabilizing the protein of their gene target ( Wilsuz et al., 2009, Scheuermann and Boyer, 2013; Marchese and Huarte, 2014). In our model, LNA mediated Thybe1 decrease does not affect the lncRNA transcription, but it down-regulates Klhl14 mRNA. Hence, it is not its transcription per sé to promote Klhl14 expression, but it could act in cis enhancing transcription factors binding to klhl14 promoter (Boque-Sastre et al., 2015). Moreover, Thybe1 knocking-down also reduces mRNA levels of several thyroid markers, suggesting Thybe1 works, *in trans*, on the targeted mRNAs. Indeed, if Thybe1 acted

binding the proteins, we would not see negative effects on mRNAs. Thybe1 could promote its target translation and/or prevent their degradation, or act as a guide for the transcription complex on the targeted promoters. Furthermore, different transcripts could play different roles. Indeed, we identified several and not reported transcripts and our preliminary data show that previously uncharacterized thyroid transcripts exist, suggesting the possibility that this lncRNA could play a very wide range of roles. This issue is still unexplored and remains a very interesting topic to deepen. Finally, to shed lights on Thybe1 role in thyroid differentiation, we are generating a conditional knockout mouse that will lost Thybe1 expression only in thyroid gland, that will allow us to study the resulting phenotype to clarify its exact role in gland development.

## 6 Conclusions

In this work we characterized for the first time a novel long non-coding RNA involved in thyroid differentiation that we named Thybe1. More interesting, we demonstrated Thybe1 is dramatically repressed by thyroid carcinogenesis highlighting an essential role of this long non-coding RNA in maintaining thyroid differentiation state.

All these data prompt to deepen the knowledge about Thybe1 functions and its implication in thyroid development and carcinogenesis. Indeed, our data hint it could represent an early marker of both thyroid specification and neoplastic transformation. Hence, its alterations, during organogenesis, could lead to different thyroid development defects implicating many clinical outcomes such as congenital hypothyroidism. Furthermore, although further studies are required to clarify this lncRNA role in thyroid differentiation and transformation processes, clinical investigations could elucidate if Thybe1 could cover a prognostic role in thyroid cancer. For these reasons it could be very interesting and useful to understand molecular mechanisms and all the possible clinical correlation and implications of this novel candidate gene playing a role in thyroid differentiation and cancer.

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